Adenovirus-Mediated p53 Gene Transfer in Patients With Advanced Recurrent Head and Neck Squamous Cell Carcinoma

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Purpose: **Standard therapies of head and neck squamous cell carcinoma (HNSCC) often cause profound morbidity and have not significantly improved survival ov r the last 30 years. Preclinical studies showed that ad noviral vector delivery of the wild-type p53 gene reduced tumor growth in mouse xenograft models. Our purpose was to ascertain the safety and therapeutic potential of adenoviral (Ad)-p53 in advanced HNSCC.**

Patients and Methods: **Patients with incurable recurrent local or regionally metastatic HNSCC received multiple intratumoral injections of Ad-p53, either with or without tumor resection. Patients were monitored for adverse events and antiadenoviral antibodies, tumors were monitored for response and p53 expression, and body fluids were analyzed for Ad-p53.**

Results: **Tumors of 33 patients were injected with doses of up to 1×10^{11} plaque-forming units (pfu). No dose-limiting toxicity or serious adverse events were noted. p53 expression was detected in tumor biopsies despite antibody responses after Ad-p53 injections. Clinical efficacy could be evaluated in 17 patients with nonresectable tumors: two patients showed objective tumor regressions of greater than 50%, six patients showed stable disease for up to 3.5 months, and nine patients showed progressive disease. One resectable patient was considered a complete pathologic response. Ad-p53 was detected in blood and urine in a dose-dependent fashion, and in sputum.**

Conclusion: **Patients were safely injected intratumorally with Ad-p53. Objective antitumor activity was detected in several patients. The infectious Ad-p53 in body fluids was asymptomatic, and suggests that systemic or regional treatment may be tolerable. These results suggest the further investigation of Ad-p53 as a therapeutic agent for patients with HNSCC.**

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SQUAMOUS CELL CARCINOMAS of the head and neck (HNSCC) comprise approximately 4% of cancers in the United States and cause approximately 2% of all US cancer deaths. The principal cause of death among these patients is local-regional recurrence.[1,2] Standard therapy is frequently associated with profound speech, swallowing, and cosmetic morbidities. Although treatment advances have been made in the last 30 years, little or no survival improvement has been obtained.[3,4] New strategies are clearly needed.

The p53 tumor-suppressor gene is the most frequently mutated gene identified in human cancers, and is mutated in a majority of HNSCs.[5] p53 is a multifunctional protein that, among other activities, acts as a transcriptional activator and repressor, is induced by DNA damage, and interacts with proteins involved in DNA replication and repair.[6,7] p53 appears to have a vital role in the sensing and repair of DNA damage, inhibiting the cell cycle to allow DNA repair, or inducing apoptosis to eliminate severely damaged cells.[7]

An adenoviral vector system was chosen for a gene therapy approach because of the ability to infect many cell types, both quiescent and dividing, lack of integration into the host genome, high-level transgene expression, ease of high titer and large scale manufacture, and the established safety of adenovirus vaccines.[8,9] The vector used is com-

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posed of the wild-type p53 gene inserted into a first-generation adenoviral backbone (Ad-p53).

Preclinical studies with Ad-p53 have shown that p53 transduction can induce apoptosis and decrease cell proliferation in a number of cancer cell lines without adversely affecting normal cells.[10-13] In general, p53 gene therapy is more effective with p53 mutant cancer cell lines, but it is also active against wild-type p53 cancer cell lines.[14-17] Ad-p53 also reduces tumor growth in xenograft models of HNSCC and other cancers.[18-20] In addition, Ad-p53 potentiates cytotoxic chemotherapy and radiation therapy in model systems.[21-23]

This study was conducted to determine the safety of Ad-p53 in patients with advanced, recurrent HNSCC; to document possible antitumor activity; and to evaluate transgene expression, Ad-p53 dissemination, and shedding. The potential suitability of direct intratumoral injections of Ad-p53 was investigated in patients with advanced disease and, in one arm of the study, in a surgical adjuvant setting.

PATIENTS AND METHODS

Study Subjects

Thirty-four patients with advanced recurrent or refractory squamous cell carcinoma of the upper aerodigestive tract (Eastern Cooperative Oncology Group performance status ≤ 2) were entered onto either the resectable or nonresectable arm of the study (Table 1). Patients were entered onto the resectable arm if the tumor could be resected for debulking, but resection alone posed no chance for a cure. All patients were presented in the M.D. Anderson Multidisciplinary Head and Neck Oncology Planning Conference before they consented to this protocol. One patient withdrew before treatment and was excluded from the analysis. Nine women and 24 men, of whom 15 had resectable disease and 18 had nonresectable disease, participated in the study. They had a mean age of 54 years (range, 32 to 76 years), and 29 patients were white, two patients were black, and two patients were Hispanic. Twenty-seven patients had had prior surgery, 32 had received prior radiation, and 21 had received salvage chemotherapy (Table 1). Patients had large tumor burdens, and the vast majority had more than one lesion. Fourteen tumors from patients in the resectable arm and 12 tumors from patients in the nonresectable arm were bidimensionally measured by computed tomographic (CT) scan before the date of the first dose. The mean area of tumors in the resectable arm was 13.76 ± 13.01 cm^2 (range, 1.00 to 38.25 cm^2; n = 12) and the mean in the nonresectable arm was 17.93 ± 16.15 cm^2 (range, 4.84 to 54.60 cm^2; n = 14). The two-sample t test showed no statistically significant difference between these two groups with regard to tumor size ($P = .4811$). The photograph (Fig 1) shows a typical advanced recurrent-disease patient entered onto this trial, and shows the absence of curative therapeutics available to these protocol patients.

Tumor p53 gene status, either mutant or wild-type, was not an entry requirement but was determined for each patient. All women of childbearing age had negative pregnancy tests, and all patients were required to practice contraception while on the study. This protocol was reviewed and approved by the Institutional Surveillance Committee of the University of Texas M.D. Anderson Cancer Center, the National Institutes of Health Recombinant DNA Advisory Committee, and the Food and Drug Administration. Informed consent was obtained from all patients before entry onto the study.

Ad-p53

Ad-p53, designated as INGN 201, is a replication-defective adenovirus serotype 5 (Ad5) vector with a p53 cDNA expression cassette that replaces the E1 region of the virus.[24] Ad-p53 is a biosafety level 2 (BL-2) agent and was handled with the appropriate or even greater level of biologic containment. Ad-p53 was produced under Good-Manufacturing-Practices conditions at Magenta, Inc (now MA Biosciences, Rockville, MD) and stored at -80°C at concentrations of 2 to 3.5×10^{10} plaque-forming units (pfu) per mL in phosphate-buffered saline supplemented with 10% glycerol. Administered Ad-p53 was free of replication-competent adenovirus at one part in 10^9 (data not shown). Ad-p53 was thawed and diluted in phosphate-buffered saline at 4°C within 2 hours of use.

Patient Treatment and Examination

Patients were sequentially enrolled at each dose level, and each patient received an assigned dose throughout the study. The dose of Ad-p53 was escalated in log increments from 10^6 to 10^9 pfu, and in half-log increments from 10^9 to 10^{11} pfu. Three to six patients were assigned to each dose level. All patients in the study received Ad-p53 therapy.

Each patient received at least one course of Ad-p53 injections. Each course consisted of Ad-p53 administration three times per week (every other day) for 2 weeks for a total of six administrations. Resectable-disease patients received only one full course of injections followed by two additional administrations; one during surgery after gross tumor removal in the site of microscopic residual disease and one 72 hours after surgery through retrograde catheter instillation. Resectable-disease patients were then observed throughout their follow-up, with no additional cycles of Ad-p53 administration. Nonresectable-disease patients underwent a 2-week rest period before the next course of Ad-p53 injections. Nonresectable-disease patients repeated the Ad-p53 courses monthly until disease progression or consent was withdrawn, for up to seven courses of treatment. Only a single site of disease was selected as the indicator lesion, even for patients with multiple sites of disease. Because the patients clearly understood the phase I nature of this investigation and the primary end points of toxicity and tolerance, efforts to inject recurrences were directed at the most accessible, assessable, and measurable masses.

Ad-p53 was injected directly into tumors, either visually or as directed by manual palpation, in a total volume of 1.5 to 10 mL, which depended on tumor volume. Patients remained under close observation for at least 2 hours after each administration. Ad-p53 injections were performed in a hospital room under respiratory and body-secretion isolation during each 2-week cycle, and the medical staff used reverse isolation procedures, which included the use of a HEPA-filtered mask. Patients remained under respiratory isolation for the 2-week investigation period until 72 hours after the last injection. Tumors were injected with a total volume based on the number of injection sites, which were spaced in 1-cm increments over the clinically assessed indicator lesion. Between 0.5 and 1.0 mL of Ad-p53 was delivered to each injection site, based on the third dimension (depth) of the mass as determined by clinical assessment.

Vital signs, hematology, chest radiography, blood chemistry, and performance status were monitored at the beginning of each treatment cycle. All adverse events reported during the study were evaluated and graded on a scale of 1 to 4. The National Cancer Institute Common Toxicity Criteria (NCI-CTC)[25] were used to determine the grade for all toxicity listed on the scale. For adverse events not listed on the NCI-CTC, the following system was used: grade 1, mild; grade 2, moderate; grade 3, severe; and grade 4, life-threatening. Ad-p53

Table 1. Patient Profile

Patient No.*	Age (years)	Sex	p53 Mutations†	p53 Immuno-staining	Prior Failed Therapies	Primary Cancer	Site of Injection	Treatment Courses‡	PFU Per Injection	Study Arm	Clinical Activity
1	32	F	arg175 to his	+	Surgery, xrt, chemo	Floor of mouth	Neck	2	10^6	R	NA
2	59	M	arg267 to pro	+	Surgery, xrt	Larynx	Neck	2	10^6	R	NA
3	57	M	ser127 to tyr	+	Surgery, xrt, chemo	Pyriform sinus	Left neck mass	1	10^6	NR	PD
4	43	F	WT	+	Surgery, xrt	Base of tongue	Right neck mass	2§	10^6	NR	PD
5	73	M	WT	−	Surgery, xrt	Unknown	Left neck mass	1	10^7	R	CR
6	46	M	leu257 to gln	+	Surgery, xrt, chemo	Cervical, esophagus	Suprastomal lesion	1	10^6	NR	SD
7	64	M	WT	−	Xrt, chemo	Tonsil	Left neck mass	5§	10^7	NR	SD
8	47	M	arg248 to pro	+	Surgery, xrt, chemo	Base of tongue	Base of tongue	1	10^7	R	NA
9	58	M	WT	+	Surgery, xrt, chemo	Larynx	Peristomal area	1	10^7	NR	PD
10	58	M	arg282 to trp	ND	Surgery, xrt	Larynx	Left hypopharynx mass	1	10^8	R	NA
11	49	M	tyr236 to cys	+	Xrt, chemo	Base of tongue	Base of tongue	1	10^8	R	NA
12	57	F	arg175 to his	+	Surgery, xrt, chemo	Floor of mouth	Left floor of mouth, mandible	3	10^8	NR	SD
13	66	M	WT	+	Surgery, xrt	Base of tongue	Right tongue, right posterior tongue	7	10^9	NR	PD
14	49	F	gln167 to stop	−	Surgery, xrt	Floor of mouth	Floor of mouth	2§	10^9	NR	PD
15	64	M	WT‖	−	Surgery, xrt, chemo	Mandible alveolar ridge	Left facial mass	1	10^9	NR	NE
16	76	F	arg248 to trp	+	Xrt	Larynx	Left supraclavicular mass	1	10^9	R	NA
17	56	M	WT	+	Surgery, xrt, chemo	Larynx	Base of tongue, left BOT, tonsil	1	10^9	NR	PD
18	57	M	WT	−	Exp	Left lateral pharyngeal wall	Left facial mass	4	10^9	NR	SD
19	54	M	WT	−	Surgery, xrt, chemo	Unknown	Right submental mass	1	3×10^9	R	NA
20	56	M	WT	−	Surgery, xrt, chemo	Tongue	Left neck mass	3	3×10^9	NR	SD
21	67	M	NE	ND	Xrt, chemo	Base of tongue	Right infraaurical area	5	3×10^9	NR	PD
22	57	M	lys132 to asn	+	Surgery, xrt, chemo	Larynx	Anterior neck, suprastomal	3	10^{10}	NR	PD
23	38	M	WT	+	Surgery, xrt	Left mandible	Left infraauricular region	6	10^{10}	NR	≥ 50% tumor regression
24	50	M	WT	−	Surgery, xrt, chemo	Left retromolar trigune	Left cheek	1	10^{10}	NR	PD
25	57	M	tyr126 to cys	+	Surgery, xrt, chemo	Pharynx	Neopharynx	1	3×10^{10}	R	NA
26	49	F	NE¶	−	Surgery, xrt, chemo	Left oral tongue	Left lateral tongue	1	3×10^{10}	R	NA
27	67	M	cys275 to trp	+	Xrt	Left tonsil	Left tonsil	1	3×10^{10}	R	NA
28	36	M	2 bp deletion at codon 209#	ND	Surgery, xrt	Base of tongue	Left base of tongue	1	10^{11}	R	NA
29	34	F	NE	ND	Surgery, xrt, chemo	Submental area	Submental area, base of tongue	1	10^{11}	R	NA
30	32	M	WT	+	Surgery, xrt, chemo	Left superior anterior neck dermal metastasis	Left anterior superior neck dermal mass	3	10^{11}	NR	≥ 50% tumor regression
31	56	M	ala 307 to ser	−	Surgery, xrt, chemo	Right hypopharynx	Right hypopharynx mass	1	10^{11}	R	NA
32	72	F	ile 232 to ser, gln 331 to stop	+	Surgery, xrt	Left buccal mass	Left buccal mass	1	10^{11}	R	NA
34	47	F	WT	+	Surgery, xrt, chemo	Nasopharynx	Right preauricular mass	4	10^{11}	NR	SD

Abbreviations: +, positive; −, negative; WT, wild-type; NE, could not be evaluated; ND, not determined; xrt, radiation therapy; NA, not applicable; NR, nonresectable; R, resectable; PD, progressive disease; SD, stable disease; chemo, chemotherapy; Exp, experimental plant extract treatment in Europe; BOT, base of tongue; CR, complete histologic response (see text).

*Patient 33 withdrew consent before the start of treatment.

†With the one noted exception, samples listed as NA could not be assessed because of insufficient tumor cells (< 10%) in the biopsy.

‡Each course had 6 injections. Several patients had a partial final course of 1-3 injections.

§The full set of injections was not completed in the last course.

‖Exon 6 was not sequenced.

¶There was only sufficient tumor to sequence exon 6; it was wild-type.

#The resulting frameshift led to a stop codon at codon 214.

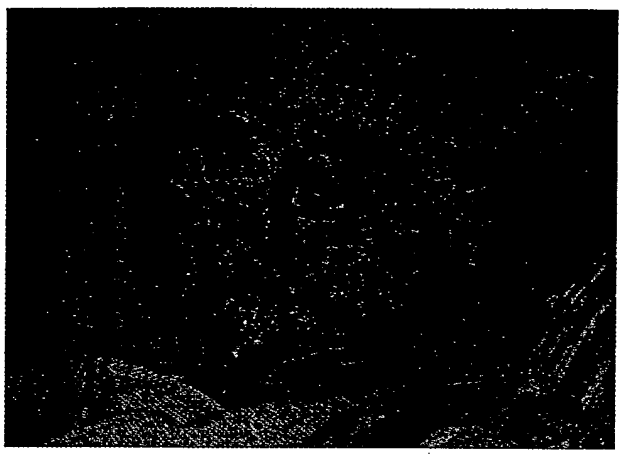

Fig 1. An aggressive recurrent squamous carcinoma of the tongue with direct extension into the neck (patient 1, resectable-disease group), which is representative of the advanced burdens of local-regional disease incurred by patients who entered onto the trial.

administrations were terminated for disease progression or inability to tolerate treatment. To avoid the enrollment of more patients than necessary onto the trial if excessive toxicity was found, a Bayesian early-stopping rule was included in the study protocol but was not implemented.

Clinical activity was evaluated by CT scan. Only nonresected patients were evaluated, by using standard criteria applied to the indicator lesion.

Sequencing of p53

Patient tumor p53 status was determined by sequencing exons 5 through 10 of the p53 gene obtained from tumor cell DNA. DNA was isolated from biopsies with a Qiagen Blood and Tissue Kit (Qiagen, Santa Clarita, CA) and polymerase chain reaction (PCR) sequencing was performed with either the AmpliCycle sequencing kit (Perkin Elmer, Norwalk, CT) or the ThermoSequenase terminator cycle sequencing kit (Amersham, Arlington Heights, IL).[26] This method allowed detection of p53 mutations if the biopsy contained 20% or greater of tumor cells.

Ad-p53 Dissemination Assays

Samples. The presence of Ad-p53 in urine, blood, and upper aerodigestive tract secretions (UATS) was assayed by cytopathic effect (CPE) and Ad-p53–specific PCR. Urine samples consisted of a first morning void that began with a pretreatment sample on the first day of treatment. Samples were collected daily during hospitalization and less regularly after discharge. Plasma was collected from Cell Preparation Tubes (Becton-Dickinson, San Jose, CA) within 4 hours of collection. Serum was obtained by standard methods. UATS consisted of expectorated sputum or saliva or a saline rinse in patients with xerostomia.

CPE assay. CPE assays were performed on 293 and A549 cells; 293 cells[27] are permissive for Ad-p53 growth and A549 cells are nonpermissive for Ad-p53 growth but permissive for growth of replication-competent adenovirus. No assays were positive for A549 CPE, which showed the lack of replication-competent adenovirus in patient samples. Plates were examined for CPE for up to 9 days. Standard curves with wild-type Ad5 (on 293 cells) and Ad-p53 (on A549 cells) were run with each assay. The CPE assay was found to be semiquantitative, with a probable error of plus or minus one order of magnitude and a sensitivity of 10 pfu per 0.5-mL sample or less. Selected 293 cell–positive CPE supernatants were assayed by an adenovirus hexon enzyme-linked immunosorbent assay (ELISA) and Ad-p53–specific PCR. Within the assay sensitivity limits, all supernatants were positive, which confirmed the identity of Ad-p53 in the patient samples.

Urine sample preparation for CPE assays included one freeze-thaw cycle, sterile filtration, and a twofold dilution in DMEM HG (Life Technologies, Gaithersburg, MD) before overlaying onto cells. After 30 minutes, two volumes of medium were added, and the plates were incubated and read as above. Patient plasma was added to 293 and A549 cells undiluted and unfiltered and scored as above. UATS samples were frozen and thawed, and homogenized vigorously by repeated pipetting and vortexing. Samples were diluted 25% to 50% with phosphate-buffered saline, clarified and sterilized with a prefilter/membrane combination (0.2-μm Serum Acrodisk filter, Gelman Sciences, Ann Arbor, MI), added to cells, and scored as above.

The hexon ELISA (Adenoclone EIA; Meridian Diagnostics, Cincinnati, OH) included controls provided by the manufacturer as well as supernatant from 293 cells infected with Ad-p53.

PCR assay. PCR was used to assay plasma, serum, urine, and UATS directly. Extreme precautions were taken to prevent PCR contamination and no evidence of such contamination was encountered. Sensitivity of the assay was 10^3 pfu per 0.5-mL sample. Oligonucleotide primers were obtained from Oligos, Etc (Wilsonville, OR).

Urine samples for PCR were frozen and thawed once, and DNA was isolated with the QIAamp HCV Kit (Qiagen). The QIAamp Tissue Kit was used to isolate DNA from 293 CPE supernatants. Primers consisted of 5′-TAGAGCCAAACTCAGCGCGG-3′ and 5′-ATCCGTGGGCGTGAGCGCT-3′. The PCR product was selected to cross a p53 open-reading–frame/adenoviral DNA junction specific for Ad-p53.

Two different blood fractions were assayed for Ad-p53. In the original protocol, serum was tested (patients 1 to 25). However, blood fractionation studies performed during the course of this trial showed enhanced sensitivity with plasma (data not shown). For patients 25 to 28, both plasma and serum were assayed. After showing equivalent results with the two assays, the remaining samples (patients 29 to 34) were assayed by using only plasma.

For PCR on serum samples, DNA was isolated with either a QIAamp Blood Kit and PCR-amplified as described above for urine, or a modification of the protocol of Cunningham et al[28] and amplified with primers 5′-CACTGCCCAACAACACCA-3′ and 5′-GCCACGCCCACACATTT-3. The PCR product was selected to cross a p53 open-reading–frame/adenoviral DNA junction specific for Ad-p53.

Plasma samples for PCR were frozen and thawed and homogenized vigorously by repeated pipetting and vortexing. DNA was extracted (QIAamp Blood Kit) and PCR-amplified as described above for urine PCR.

UATS samples for PCR were frozen and thawed, homogenized vigorously by repeated pipetting and vortexing, and DNA was extracted with the QIAamp Tissue Kit and PCR-amplified as described above for urine PCR.

Reverse-transcriptase PCR. The presence of p53 transgene mRNA in patient biopsies was detected by reverse-transcriptase (RT)-PCR. Total RNA was isolated with TRI reagent (Molecular Research, Cincinnati, OH) from pre- and posttreatment flash-frozen biopsies. The RNA was then DNAse I-treated and reverse transcribed (Superscript RNAse H^-RT and random hexamer primers [Life Technologies]), and the resulting DNA amplified by PCR by using primers specific to Ad-p53 mRNA. (5′-GGTGCATTGGAACGCGGATT and 5′-GGGGACAGAACGTTGTTTTC). Identity of the PCR products was confirmed

by Southern blot hybridization.[26] Positive controls for glyceraldehyde-phosphate dehydrogenase (GAPDH) mRNA and negative controls (blanks, minus RT) gave the expected results (data not shown).

Anti-Ad5 Antibody Assays

Serum samples were tested for anti-Ad5 immunoglobulin G by Virolab, Inc (Berkeley, CA) by an indirect immunofluorescence assay.[29]

Histology

Hematoxylin-and-eosin stained slides from each biopsy were prepared and evaluated for histologic grade, extent of viable tumor and necrosis, and the presence of inflammatory cell infiltrates.

Immunohistochemical staining (IHC) for p53 was performed on formalin-fixed, paraffin-embedded tissue sections by DO-1 antibody (Oncogene Science, Uniondale, NY) with an avidin-biotin-peroxidase complex method.[30] Scoring was accomplished by counting positive nuclear staining in 100 to 200 tumor cells in 10 consecutive high-power fields. Specimens were scored for the percentage of cells that expressed p53. All slides were coded, evaluated, and scored in a blinded fashion. Samples with 20% or greater of tumor cells that showed positive staining were defined as IHC-positive.

RESULTS

Treatment and Safety

A nonrandomized, phase I, dose-escalation study was conducted to ascertain the safety of Ad-p53 in resectable and nonresectable HNSCC patients. Efficacy and biodistribution were also monitored. Doses ranged from 10^6 to 10^{11} pfu, with six to 42 doses of Ad-p53 administered per patient over a course of 2 weeks to 6½ months. A total of 429 doses of Ad-p53 were administered, with a maximum total-dose per patient of 3×10^{12} pfu.

The multiple courses of direct intratumoral injections of Ad-p53 were well tolerated. Neither dose-limiting effects of Ad-p53 injection nor serious adverse events related to the study treatment occurred. Injection site pain was the most common adverse event and occurred in 19 patients, but it did not correlate with dose or anatomic site of injection. Other Ad-p53–related adverse events that occurred three or more times were seen primarily at doses of 10^{10} pfu or greater (14 of 17 events) and consisted of transient fever, headache, pain, and edema. Injection site pain and headache resolved within 24 hours, fever within 48 hours, and edema within 4 days. There was mild erythema at the site of injection with doses of 3×10^9 pfu or greater, which did not alter treatment schedules. No evidence of systemic hypersensitivity or allergic reaction was seen despite patients who received as many as seven monthly courses at 10^9 pfu, six courses at 10^{10} pfu, or four courses at 10^{11} pfu. Peri- and postoperative administration of Ad-p53 had no adverse effect on wound healing (data not shown).

Because health care providers were potentially exposed to Ad-p53 during injections, the two providers with the greatest risk of exposure were tested. Serum collected after more than 75% of the doses had been administered showed low levels of anti-Ad5 antibody and a lack of infectious Ad-p53 or Ad-p53 DNA. The level of antibody determined in sera from health care providers was within the range of all pretreatment serum values determined for all patients in the study. Urine also contained no infectious Ad-p53 or Ad-p53 DNA. These findings suggest no significant exposure of health care providers to Ad-p53 during this study.

Patient p53 Mutational Status

Patient p53 mutational status was determined both by sequencing of genomic DNA (exons 5 through 10) and by IHC. Generally, wild-type p53 protein 15 was present at very low levels that were undetectable by IHC, whereas mutant p53 protein 15 was present at much higher levels and can be detected by IHC.[31] Overall, 58% of patients were p53 mutant as determined by IHC and 48% were p53 mutant as determined by sequencing (Table 1). Agreement was observed between IHC and sequencing in 71% of the patients who could be evaluated. Patient 14 showed a mutation that led to a truncated p53 protein that could not be detected by IHC.

Clinical Results

Ad-p53 administration resulted in objective tumor regressions (Table 1) in some refractory- or recurrent-disease patients despite large tumor burdens. Of the 17 nonresectable-disease patients, two showed a greater than 50% reduction in the indicator lesion by CT scan, six showed stable disease, and nine progressed. The two patients who showed the greatest activity received doses of 10^{10} and 10^{11} pfu (Figs 2 and 3). The duration of clinical activity was 7 weeks in one patient and was documented for 18 days in the second patient, at which point the patient withdrew from the study and was lost to follow-up. The duration of stable disease in six patients was 1 to 3.5 months. Several nonresectable-disease patients showed tumor progression in nonindicator lesions, which caused morbidity and removal from the study.

Resectable-disease patients were not considered assessable for antitumor activity because of removal of the indicator lesion. However, patient 5 (treated with six doses of 10^7 pfu) showed a complete pathologic response in that no viable tumor was found in the completely resected specimen. At the time of pathologic evaluation, the tumor mass had been replaced by liquifactive necrosis. This patient remains disease free at 26 months from study entry. Resected-disease patient 10 also showed no evidence of disease at 24 months from study entry. At the time of this publication, six of the 15 resected-disease patients have died, which included one non–cancer-related death.

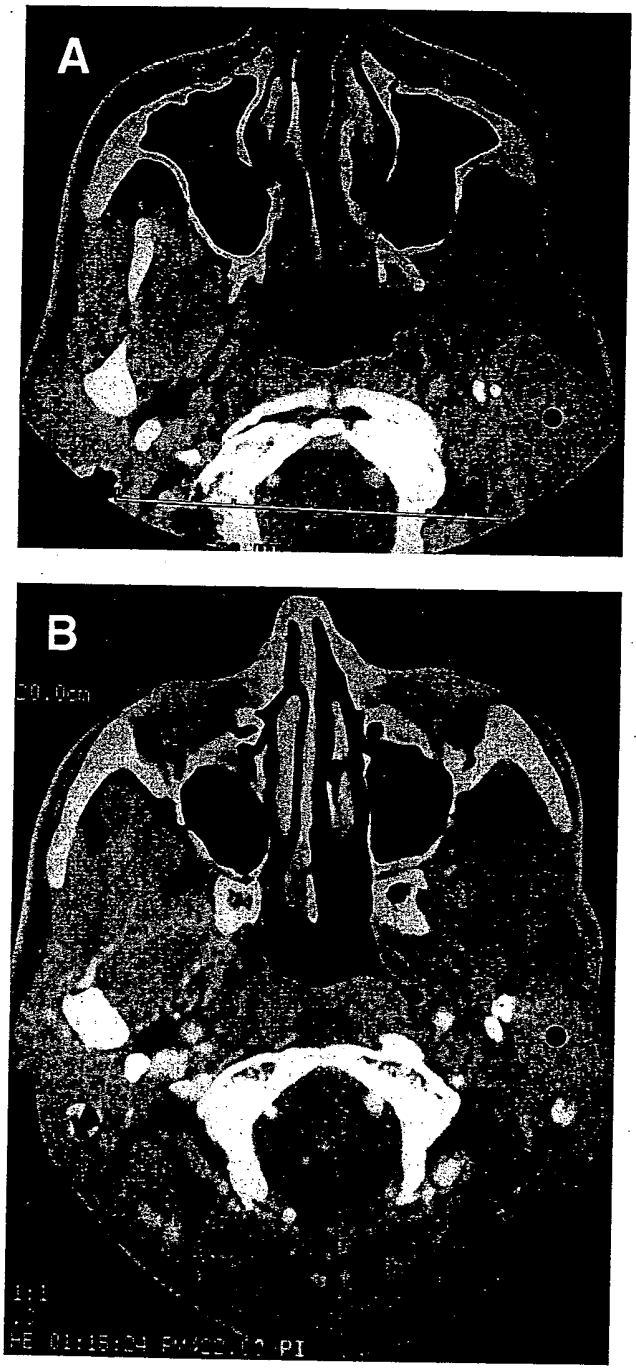

Fig 2. Axial post-contrast CT images (before and after 6 doses of 10^{10}) in patient 23, with recurrent HNSCC and previous hemimandibulectomy and flap reconstruction. (A) Pretreatment, postoperative distortion and enhancing tumor in the left condylar fossa/subtemporal region (dot). (B) Posttreatment, > 50% tumor reduction (dot).

The median survival time for all treated patients was 267 days and for all resectable-disease patients was 408 days. Median survival time for the nonresectable-disease patients was 127 days, which was consistent with other reported phase I and II studies of advanced recurrent head and neck cancer.[1,4,32] Of the 18 nonresectable-disease patients, 15 have died. However, death does not necessarily reflect progression of the lesions injected with Ad-p53 because progression also occurred at untreated sites.

Ad-p53 Transduction

Expression of the p53 transgene (Table 2) was detected by RT-PCR from patients 10 and 13 from biopsy samples taken at 4 and 48 hours after treatment, respectively (Fig 4). In contrast, biopsies from patients 5 and 8 at 1 hour after Ad-p53 delivery were negative. Tissues collected from non–Ad-p53-injected sites were used as controls. The positive biopsy sample from patient 13 was taken 67 days after the start of Ad-p53 injections (and 48 hours after the last injection) and showed transgene expression long after the development of a strong antibody response to Ad5. No transgene mRNA was detected in pretreatment biopsy samples or in non–Ad-p53-injected tumor samples (data not shown).

p53 IHC analysis was complicated by the large proportion of patients (52%) with high-positive (> 50% of cells) pretreatment values for p53. In a subset of 12 patients with pretreatment p53 values of 50% or less, three patients showed increased p53 protein expression after treatment. Figure 5 shows an increase in p53 IHC staining 48 hours after the last Ad-p53 injections compared with the prestudy biopsy sample.

Immune Response

All patients injected with doses greater than 10^7 pfu showed an increase in antiadenovirus type 5 antibody. All increases were manifested by 4 weeks, although a more limited sampling suggested the increase occurred by 1 week. The largest increase in titer was 2,048-fold greater than the prestudy levels. Antibody induction level did not correlate with Ad-p53 dose or course of treatment (data not shown).

Biodistribution

Ad-p53 DNA was detected in blood in a dose-dependent manner, as assayed by PCR (Table 3). Using this method, Ad-p53 DNA was present in blood by 30 minutes after Ad-p53 injections and was absent by 48 hours (data not shown). CPE data from four patients treated at 3×10^{10} and 10^{11} pfu showed viable Ad-p53 present at the highest levels 30 minutes after injections, a decrease of two to four orders of magnitude by 90 minutes, a further decrease to a very low or undetectable titer by 24 hours, and an absence by 48 hours after injections.

Infectious Ad-p53 was detected in urine from some patients who received doses of 3×10^9 pfu or greater and was present in urine from all patients who received doses of

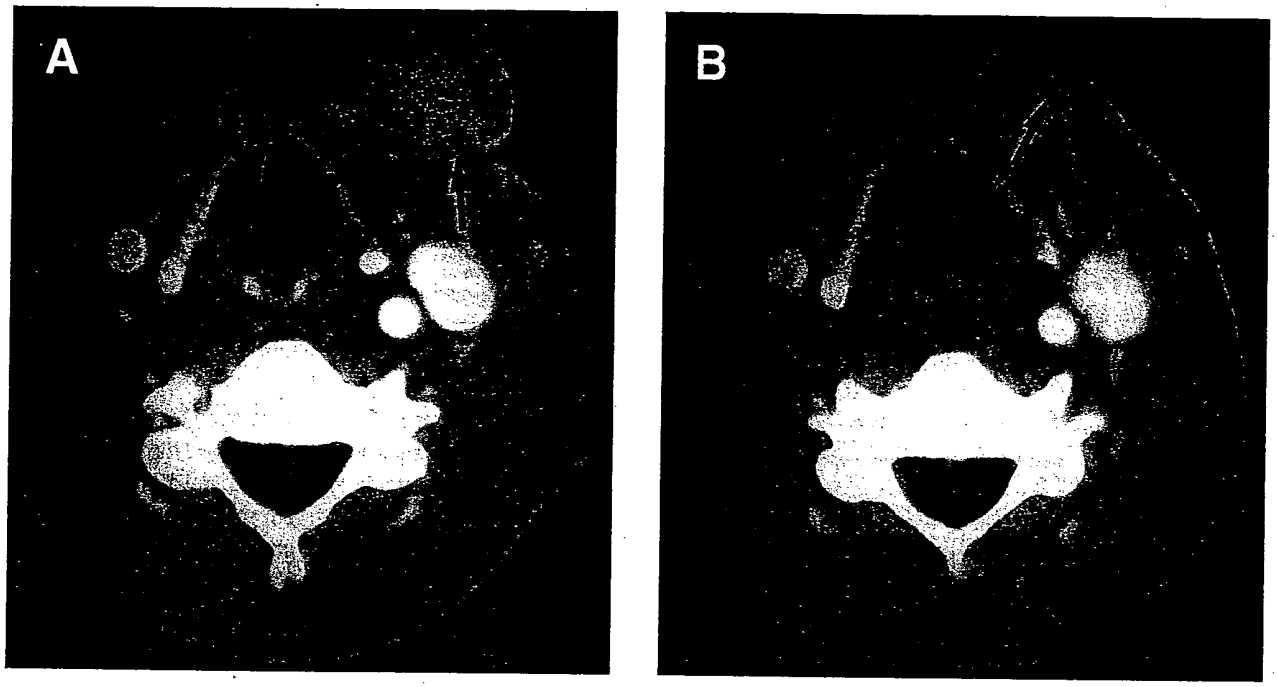

Fig 3. Axial post-contrast CT images (before and after 6 doses of 10^{11} pfu) in patient 30, with recurrent squamous cell carcinoma and previous bilateral neck dissections. (A) Pretreatment, left-sided metastatic dermal implant (arrow). (B) Posttreatment, > 50% tumor reduction (arrow).

3×10^{10} pfu or greater (Table 3). Ad-p53 was generally detected within 1 day of the beginning of Ad-p53 injections and was present throughout cycles. A representative example of the time course of Ad-p53 in urine during treatment is shown in Fig 6. The highest titer detected in urine was 10^6 pfu per 0.5 mL. Urine was free of Ad-p53 within 3 to 17 days of the last Ad-p53 injection.

Ad-p53 was also detected in sputum and/or saliva samples of the six high-dose patients tested (patients 28 through 34, who all received 10^{11} pfu). The highest titer found was 10^6 pfu per 0.5 mL. As with urine samples, Ad-p53 was generally detected within 1 day of the first injection of Ad-p53 and was present throughout each cycle. Ad-p53 was usually present for several days after the last injection of Ad-p53 and was cleared to background levels within 7 days. The time-course profiles were similar to those found for urine.

Table 2. Assay of Ad-p53 Transgene Expression by RT-PCR

Patient No.	Dose (pfu)	Time From Last Dose (hours)	Time From First Dose (days)	Anti-Ad5 Antibody*	RT-PCR
5	1×10^7	1	18	2	–
8	1×10^7	1	29	64	–
10	1×10^8	4	14	8	++
13	1×10^9	48	67	128	+

*Fold increase over pretreatment levels; ≥ four-fold represents a significant increase.

DISCUSSION

After Ad-p53 administration to patients with HNSCC, the primary consideration in this phase I clinical study was patient safety. Adverse events reported in the study were typical of this patient population. No untoward incidents or trends were noted, and no serious adverse events related to injection of Ad-p53 were detected. Thus, up to 3×10^{12} pfu of Ad-p53 could be administered without serious side effects. This level is believed to be the highest dose of adenoviral vector to be delivered to patients to date. A maximum-tolerated dose was not defined in this trial because the maximum dose of 1×10^{11} pfu led to no clinically significant side effects.

Pain at injection site was the most common treatment-related adverse event, and is believed to be related to the injection of ice-cold Ad-p53 solution. Recent stability studies indicate that chilling Ad-p53 on ice until immediately before injection is not necessary. An increase in the temperature of the injected Ad-p53 could minimize this pain in future studies.

Ad-p53 administration led to objective antitumor activity in some patients. Of the 17 nonresectable-disease patients, two showed a greater than 50% reduction of the indicator lesion, and six showed stable disease at the indicator lesion. Although resectable-disease patients could not be formally characterized for response, patient 5 had a complete pathologic response and remains disease free at 26 months.

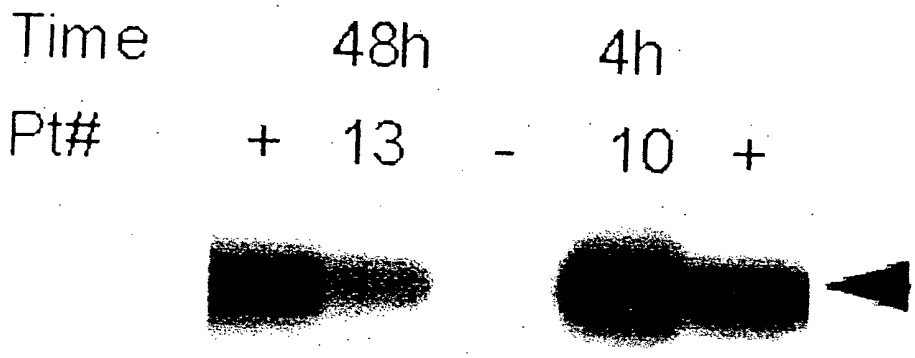

Fig 4. Demonstration of Ad-p53 transgene expression by RT-PCR and Southern blot hybridization. Patient 13 was biopsied 48 hours after treatment, and patient 10 was biopsied 4 hours after treatment. (Arrowhead), the expected size of the PCR product; (+) positive control; (−) negative control.

Patient 10 remains alive with no evidence of disease at 24 months. Only six deaths (one non–cancer related) have occurred among the 15 patients with resected disease. These responses are even more impressive given the large tumor burdens carried by most of the patients (example in Fig 1). In the nonresectable-disease patients, the Kaplan-Meier median survival time was 127 days, consistent with other phase I and phase II studies of advanced recurrent head and neck cancer.[1,4] The median survival for resectable-disease patients was 408 days, or 13.6 months, and the overall median survival was 267 days, which is about 60% longer than that reported in chemotherapy trials with a similar patient profile.[32] Although preliminary and uncontrolled, it is believed that these are encouraging data that could support evaluation of Ad-p53 in the treatment of HNSCC. These results are also in agreement with a previous study that showed that restoration of wild-type p53 expression by a retroviral vector in non–small-cell lung cancer could mediate tumor regression.[33] The design of the study does not allow us to conclude that the observed clinical activity was specifically a result of vector and transgene induction.

Patients with indicator lesion regression of 50% or more were discordant for p53 status as assayed by DNA sequencing and IHC analysis (Table 1). However, the tumors of the three patients with stable disease were wild-type for p53 as shown by both assays. In addition, patient 5, who underwent a complete histologic response and is alive with no evidence of disease at 26 months after the start of treatment, is p53 wild-type by both sequencing and IHC. These data strongly suggest that Ad-p53 can exert a significant antitumor effect on HNSCC tumors regardless of the p53 status of the tumors.

The simplest explanation for the observed responses to Ad-p53 is that p53 expression kills tumor cells. However, given previous studies on the spread of adenovirus after intratumoral injection[34] and the large sizes of the tumors in this study, one might expect a minority of tumor cells to be infected by Ad-p53. In considering other effects of Ad-p53 on the tumor, one possibility is that p53 expression may be exerting an antiangiogenic effect. Evidence that wild-type p53 decreases the expression of angiogenic factors and increases the expression of antiangiogenic factors has been presented.[35-37] Another possibility is that the adenoviral vector triggers an immune response that might adversely

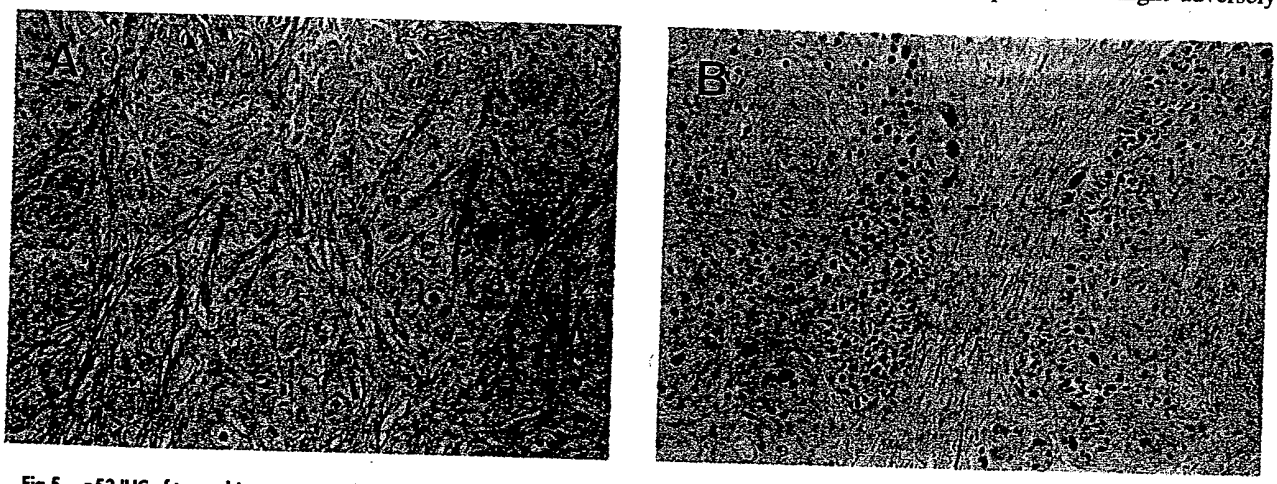

Fig 5. p53 IHC of tumor biopsy sections from patient 8 (p53 mutant, IHC positive) after 7 doses of 10^7 pfu. (A) Pretreatment biopsy. (B) Biopsy of the wound bed 48 hours after wound-bed treatment. The light to dark brown nuclear stain represents positive p53 IHC.

Table 3. Presence of Ad-p53 DNA in Body Fluids

Dose	Blood		Urine
	30-90 Minutes*	24 Hours*	
10^6	0	0	0
10^7	25	0	0
10^8	0	0	0
10^9	50	0	0
3×10^9	66	0	66
10^{10}	100	0	66
3×10^{10}	100	0	100
10^{11}	100	0	100

NOTE. Table indicates the detection of Ad-p53 DNA by PCR in serum (patients 4, 5, 7-28) and urine (all patients).

*Value indicates percentage of patients with at least one positive sample.

affect the tumor. In addition, E1-deleted, replication-defective adenoviruses can in fact replicate to some extent if the multiplicity of infection is high enough.[38] Because Ad-p53 was injected directly into tumors, the possibility of a high multiplicity of infection cannot be excluded. Local Ad-p53 replication could have several effects, which include direct killing of cells and enhancement of the host immune response.

RT-PCR showed the ability of Ad-p53 to infect tumor cells and induce expression of p53 mRNA (Fig 4). Transgene mRNA was detected in samples collected 4 and 48 hours after the last injection, but not in two samples collected 1 hour after treatment. This lack of signal in the 1-hour samples could be because of insufficient time for expression, the lower dose administered to patients in the 1-hour samples, or sample variation (see below). Interestingly, the transgene mRNA expression detected 48 hours after the last injection occurred 67 days from the start of treatment. By this time, a strong antibody response to adenovirus had occurred (Table 2), which led to the conclusion that a strong humoral immune response does not prevent transgene expression from intratumoral injection of Ad-p53.

One example of IHC that showed increased p53 expression after treatment is shown in Fig 5. Biopsies from three patients with low pretreatment p53 IHC showed increased p53 IHC after Ad-p53 injection. Several explanations are possible for the sporadic nature of the detection of increased p53 IHC after Ad-p53 injection. First, Ad-p53–infected tumor cells that undergo apoptosis are no longer present and cannot be evaluated for IHC signal. Biopsies for IHC were performed 3 days after the last injection, but recent data (not shown) suggest that earlier sampling may have detected p53 expression before cell death. Second, necrotic areas that appeared in some tumors after treatment were avoided during biopsy and histopathologic analysis. If necrotic areas were caused by Ad-p53, the biopsy procedure would be biased so as to underrepresent p53 IHC. Third, the large nature of the tumors made sampling of tumors in close proximity to sites of Ad-p53 injection difficult.

The p53 mutation spectrum in the population of treated patients was found to be consistent with earlier studies.[39] Most tumor samples (71%) analyzed for p53 status showed agreement between detection of mutations by partial genomic sequencing and IHC. This degree of concordance was also consistent with other reports.[40] The subset of patient biopsy specimens for which IHC and DNA sequencing disagreed may be because of the inability of sequencing technologies to detect a low percentage of tumor DNA in biopsy samples, mutations outside of exons 5 through 10,[39] accumulation of wild-type p53 because of MDM2 overexpression,[41] and p53 mutant proteins that do not show increased stability.[42] As evidence of the first possibility, when a tumor sample from patient 23 was screened with a more sensitive sequencing method (OncorMed, Gaithersburg, MD), a mutation was indeed detected in exon 8.

The data presented from this study indicate much more extensive biodistribution and dissemination than had previously been detected. The appearance of Ad-p53 in blood was relatively constant and appeared in almost every patient 30 to 90 minutes after injection at doses greater than 10^9 pfu. Infectious vector had not previously been detected in patient blood after the administration of up to 2×10^9 pfu to nasal and/or bronchial epithelia,[43,44] a dose level that caused detectable vector in blood in this study (Table 3).

Wild-type Ad5 had been reported to be present in urine in rare cases of acute infections in severely immunocompromised patients.[45] However, the substantial levels and duration of excretion of the replication-defective Ad-p53 in urine were remarkable. The consistent detection of Ad-p53 in urine at doses of 3×10^9 pfu or greater is in contrast to other studies[43,44,46] that did not detect vector in urine after administration of up to 2×10^{10} pfu. Detection of Ad-p53 in

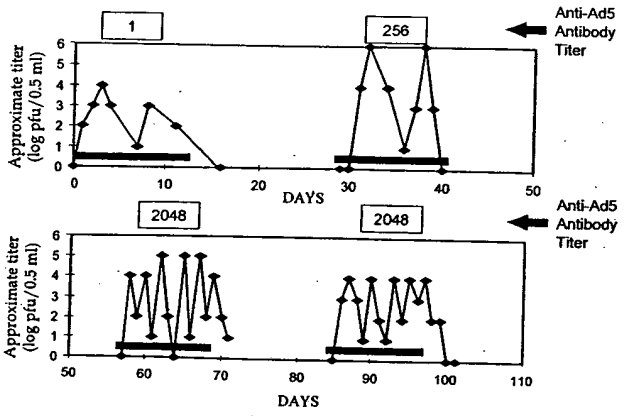

Fig 6. Presence of infectious Ad-p53 in urine from patient 34 as assayed by CPE. Day 1, first day of treatment; solid bars parallel to the X-axis mark treatment courses 1-4. Boxed numbers above the graph denote anti-Ad5 antibody titers, measured at the start of each course.

blood and urine in this study may be because of more sensitive detection methods, more facile dissemination from tumoral injection sites compared with the nasal/bronchial administration route used by others, or the low number of patients treated at high doses in other studies.

The presence of Ad-p53 in UATS samples is not surprising for patients whose tumors intrude into the oral cavity or upper airway, particularly for those patients in whom the tumor was injected through the oral cavity or airway. However, several of the tumors were not contiguous with the airway, which suggests that Ad-p53 can gain access to the airway indirectly through systemic and probably blood-borne biodistribution.

The fluctuations in urine and UATS Ad-p53 levels during courses of Ad-p53 treatment and the large variation in the amount of time necessary to return to baseline after Ad-p53 administration (1 to 17 days) may be because of heterogeneity in tumor location and size. Also, the urine and UATS samples were heterogeneous in that the urine samples were first morning voids and not full 24-hour collections and the UATS samples were either sputum, saliva, or oral cavity wash, which depended on the patient.

As expected, almost all patients developed anti-Ad5 antibodies over the course of treatment. This response did not seem to be deleterious to the patients. In addition, the patient immune response did not block transduction and expression of the p53 transgene. Numerous animal studies with replication-defective adenovirus have shown decreased transgene expression as a result of an immune response. Furthermore, these studies suggested a total lack of transduction as a result of repeated doses that were also believed to be because of an immune response.[47,48]

The patient immune response also did not prevent the appearance of Ad-p53 in blood, urine, and UATS (Fig 6, data not shown). Whereas it is known that patients may shed wild-type adenovirus for many months after an infection and in the presence of a humoral immune response,[45,49] it was originally hypothesized that dissemination would be affected at least to some effect by an immune response. However, the previous studies involved intravenous administration of adenoviral vectors in mice, which may not be relevant to this study. First, intratumoral injection may differ substantially from systemic administration. Bramson et al[50] recently showed efficient transgene expression after administration of an adenoviral vector by intratumoral injection in mice previously immunized with Ad5. Therefore, tumors may represent immunologic sanctuaries. Second, the use of mice as a model organism may be problematic, because the biologic activity of adenovirus in humans and mice is very different in that mice are not permissive for Ad5 replication.[51]

The promising results noted in this study suggest that Ad-p53 may show activity in patients with HNSCC; therefore, further study is needed and is underway. Established patient benefit is not clearly or routinely evident. Possible applications are nevertheless attractive. Brennan et al[52] used a sensitive PCR-based assay to detect tumor-specific p53 mutations in resected tumor margins from patients whose tumor margins were histopathologically free from residual tumor. They found that patients with molecular evidence of tumor cells (as detected by tumor-specific p53 mutations) have a higher likelihood of recurrence and mortality. Positive RT-PCR results that showed transduction in margins after surgery in this study (patient 10; Table 2) give direct evidence that surgical intervention may allow gene transfer into surgical sites of microscopic tumor. Given that peri- and postoperative administration of Ad-p53 had no adverse effect on wound healing, this approach may be valuable as adjuvant therapy in areas of microscopic residual disease at tumor margins to prevent recurrence and avoid further surgical ablation of normal tissue. The impact of gene transfer strategies additionally requires significant development in delivery methods that can effectively and efficiently distribute the vector to other lesions, mucosa, and potential microscopic disease sites.

Other possible future directions for Ad-p53 gene therapy include combination therapy with radiation or cytotoxic agents, as suggested by the enhanced antitumoral effects of combination treatments in preclinical models.[21-23] Also, the identification of p53 mutations in head and neck premalignancies[53] suggests that this approach may be therapeutic in severely dysplastic preinvasive lesions of this region.

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